

## Final Report

Study Title  
Biodegradation Test of

Author  
Kenji Miya

Study Completion Date  
December 13, 2010

Test Facility  
Nisso Chemical Analysis Service Co., Ltd.  
345 Takada, Odawara, Kanagawa 250-0216, Japan

Sponsor

Study No.  
NCAS 10-221

TRUE COPY OF ORIGINAL  
6/14/12  
Masato Sugawara

## GLP Compliance Statement

Study No.: NCAS 10-221  
Study Title: Biodegradation Test of

This study was performed in accordance with the Good Laboratory Practice Regulations of "Standard for the test facility conducting tests concerning new chemical substances, etc." (Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, No. 1121003, November 21, 2003; Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, No. 3, November 17, 2003; Environmental Policy Bureau, Ministry of the Environment, No. 031121004), Final Amendment: July 4, 2008, and OECD Principles of Good Laboratory Practice [OECD ENV/MC/CHEM (98) 17, 1998].

This study was carried out in accordance with the following procedure and the final report was prepared faithfully and consistently with the raw data obtained.

Study Director:	(signature) _____	(seal)	_____	(December 13, 2010)
	Kenji Miya			Date
	Odawara Laboratory			
	Nisso Chemical Analysis Service Co., Ltd.			

The original signature page of GLP Compliance Statement is shown in page 3.

## Original GLP Compliance Statement

The English translation of GLP Compliance Statement is shown in page 2.

報告書番号 NCAS 10-221

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### GLP 適合陳述書

試験番号： NCAS 10-221

試験名：

この試験は「新規化学物質等に係る試験を実施する試験施設に関する基準について」(平成 15 年 11 月 21 日 薬食発第 1121003 号、平成 15・11・17 製局第 3 号、環保企発第 031121004 号)、最終改正：平成 20 年 7 月 4 日および OECD Principles of Good Laboratory Practice [OECD ENV/MC/CHEM (98) 17, 1998] に従って実施した。

この試験は、ここに述べられた方法により行われ、この最終報告書は試験実施により得られた生データを正確に反映したものである。

試験責任者

宮 賢治



宮 賢治  
(株)日曹分析センター 小田原事業所

12/13/2010

日付

## Quality Assurance Statement

Study No.: NCAS 10-221

Study Title: Biodegradation Test of

Quality Assurance inspections of the study referred above were conducted according to the appropriate GLP regulations and the standard operating procedures (SOPs) of the Quality Assurance Unit (QAU). The results of the inspections were reported to the study director and the facility management on the following dates.

Items inspected	Dates (Month/Day/Year)		
	Inspected	Reported to	
		SD	Management
Study plan	9/16/2010	9/16/2010	9/16/2010
Study plan amendment No.1	10/7/2010	10/7/2010	10/7/2010
Study plan amendment No.2	11/15/2010	11/16/2010	11/16/2010
Experimental operation			
• Receipt and maintenance of the activated sludge	9/24/2010	9/24/2010	9/24/2010
• Receipt and maintenance of the activated sludge (new lot)	10/18, 19/2010	10/19/2010	10/19/2010
• Infrared resonance spectral measurement	9/17, 24/2010	9/24/2010	9/24/2010
• Preparation of the test systems for BOD measurement	9/21, 24/2010	9/24/2010	9/24/2010
• Set up of the enclosed respirometer and BOD measurement (1 <sup>st</sup> trial)	9/21, 24/2010	9/24/2010	9/24/2010
• Set up of the enclosed respirometer and BOD measurement (3 <sup>rd</sup> trial)	10/18, 19/2010	10/19/2010	10/19/2010
• BOD measurement and observation	9/28/2010	9/28/2010	9/28/2010
• BOD measurement and observation (2 <sup>nd</sup> trial)	10/7/2010	10/7/2010	10/7/2010
• BOD measurement and observation (3 <sup>rd</sup> trial)	10/25/2010	10/25/2010	10/25/2010
• Sampling and treatment of the bottle contents	11/15, 16/2010	11/16/2010	11/16/2010
• Preparation of the standard solutions (LC/MS/MS)	11/15, 16/2010	11/16/2010	11/16/2010
• Preparation of the standard solutions (AA)	11/15, 16/2010	11/16/2010	11/16/2010
• Analysis (LC/MS/MS)	11/15, 16/2010	11/16/2010	11/16/2010
• Analysis (AA)	11/15, 16/2010	11/16/2010	11/16/2010
Raw Data	11/26-30/2010	11/30/2010	11/30/2010
Draft Report	11/26-30/2010	11/30/2010	11/30/2010
Final Report	12/13/2010	12/13/2010	12/13/2010

The QAU found that the study was performed according to the study plan and SOPs, the reported methods and procedures were actually used and the results accurately reflect the recorded data.

QAU Manager: (signature) (seal) (December 13, 2010)  
Ken Watabe  
Nisso Chemical Analysis Service Co., Ltd. Date

The original signature page of Quality Assurance Statement is shown in page 5.

## Original Quality Assurance Statement

The English translation of Quality Assurance Statement is shown in page 4.

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## 信頼性保証書

試験番号: NCAS 10-221

試験名:

上記試験の信頼性保証の監査または査察を適用 GLP および信頼性保証部門 (QAU) の SOP に基づいて実施した。監査または査察の結果は、以下の日付で試験責任者および運営管理者に報告した。

監査または査察項目	日付 (月/日/年)		
	監査または査察日	報告日	
		試験責任者	運営管理者
試験計画書	9/16/2010	9/16/2010	9/16/2010
試験計画書変更届 1	10/7/2010	10/7/2010	10/7/2010
試験計画書変更届 2	11/15/2010	11/16/2010	11/16/2010
実験操作			
・ 活性汚泥の入手と管理	9/24/2010	9/24/2010	9/24/2010
・ 活性汚泥の入手と管理 (新ロット)	10/18, 19/2010	10/19/2010	10/19/2010
・ 赤外線吸収スペクトル測定	9/17, 24/2010	9/24/2010	9/24/2010
・ BOD 測定のための溶液調製	9/21, 24/2010	9/24/2010	9/24/2010
・ 閉鎖系酸素消費量測定装置 の設定と測定開始	9/21, 24/2010	9/24/2010	9/24/2010
・ 閉鎖系酸素消費量測定装置 の設定と測定開始 (再実験 2 回目)	10/18, 19/2010	10/19/2010	10/19/2010
・ BOD 測定および観察	9/28/2010	9/28/2010	9/28/2010
・ BOD 測定および観察 (再実験)	10/7/2010	10/7/2010	10/7/2010
・ BOD 測定および観察 (再実験 2 回目)	10/25/2010	10/25/2010	10/25/2010
・ BOD 測定終了後の 分析前処理	11/15, 16/2010	11/16/2010	11/16/2010
・ 標準溶液の調製 (LC/MS/MS)	11/15, 16/2010	11/16/2010	11/16/2010
・ 標準溶液の調製 (原子吸光分析)	11/15, 16/2010	11/16/2010	11/16/2010
・ 分析 (LC/MS/MS)	11/15, 16/2010	11/16/2010	11/16/2010
・ 分析 (原子吸光分析)	11/15, 16/2010	11/16/2010	11/16/2010
生データ	11/26-30/2010	11/30/2010	11/30/2010
報告書草案	11/26-30/2010	11/30/2010	11/30/2010
最終報告書	12/13/2010	12/13/2010	12/13/2010

QAU は、この試験が試験計画書および SOP に従って行われ、報告された方法や手段が実際に使われたものであり、結果は記録されたデータを正確に反映していることを確認した。

QAU 責任者




渡部 健

2010 年 12 月 13 日

(株) 日曹分析センター

## Study Information

Study No.: NCAS 10-221  
Study Title: Biodegradation Test of  
Report No.: NCAS 10-221

Sponsor:

Client:

Test Facility: Nisso Chemical Analysis Service Co., Ltd.  
Odawara Laboratory  
345 Takada, Odawara, Kanagawa 250-0216, Japan  
TEL 0465-42-8207 FAX 0465-42-3586

Study Director: Kenji Miya

Experimenter:

Confirmation of the Test Substance	Shinpei Tsushima, Hiroyuki Ueda
Confirmation of the Stability of the Test Substance	Hiroyuki Ueda
Cultivation (BOD measurement)	Hiroyuki Ueda
LC/MS/MS measurement	Atsushi Utsugi
AA measurement	Junko Tashiro

Study Initiation Date: September 16, 2010  
Experimental Start Date: September 17, 2010  
Exposure Period: October 18, 2010 — November 15, 2010  
Experimental Completion Date: November 17, 2010  
Study Completion Date: December 13, 2010

Testing Guideline: "Biodegradation Test of Chemical Substances" prescribed in "Test Methods for New Chemical Substances", Pharmaceutical and Food Safety Bureau No. 1121002 of MHLW, Manufacturing Industries Bureau No. 2 of METI and Environment Policy Bureau No. 031121002 of MOE (Amended on November 20, 2006), and OECD Guidelines for the Testing of Chemicals No. 301C (1992).

Archiving: All the documents of this study will be retained for 10 years after the end of this study in the archives of the test facility. The management of the documents after that will be determined by mutual consultation between the Sponsor and Nisso Chemical Analysis Service Co., Ltd. at that time. The test substance will be returned after the end of this study.

Deviation from SOPs and Study Plan:

There was no deviation from SOPs and the study plan.

Circumstance/matter that Affect the Reliability of the Test Result:

There was no environmental agent which influenced the reliability of the test result.

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## Summary

The biodegradation test (28 days) of \_\_\_\_\_ was conducted and the mean degradability calculated from the biological oxygen demand (BOD) was 2% (n=3). Furthermore, the mean degradabilities calculated from the residual amount of the test substance obtained by measurement with the liquid chromatograph mass spectrometer and the atomic absorption spectrophotometer were 0% (n=3) and -4% (n=3), respectively. It was considered that \_\_\_\_\_ is not ready biodegradable because the degradabilities for all vessels were less than 60% (Test Methods and Judgment Criteria for the Designation of Monitoring Chemical Substances, April 12, 2010).

## Introduction

The biodegradation test of \_\_\_\_\_ was performed to evaluate the biodegradability.

## Materials and Methods

### 1. Test Substance

Name:

Rational formula:

Molecular formula:

Molecular weight:

CAS number:

Lot No.:

Purity:

NCAS retrieval No.: STD-1180-4 (Preparation of the test systems, Identification and stability of the test substance)  
STD-1180-6 (Preparation of the standard solutions for LC/MS/MS measurement)

Supplier:

Received weight: 0.5 g × 5 bottles and 7.5 g × 1 bottle

Received date: July 6, 2010

Expiry date: May 27, 2011

Storage condition: The substance in a glass bottle was covered with the aluminum lamination bag, and was stored at room temperature (dark).

Physicochemical properties: Soluble in DMSO, acetone and water, Deliquescence

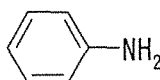
The test substance was weighed as 100% of purity.

### 2. Standard Substance

Name: Aniline

Molecular formula: C<sub>6</sub>H<sub>7</sub>N

Chemical structure:



Molecular weight: 93.13  
CAS No.: 62-53-3  
Lot No.: ALL9668  
Purity: Regarded as 100%  
NCAS retrieval No.: ANI-013  
Supplier: Wako Pure Chemical Industries, Ltd.  
Received weight: 12-mL ampoule, 25 ampoules  
Received date: August 25, 2008  
Expiry date: August 25, 2011  
Storage condition: Stored in a freezer (about -20°C)  
Appearance: Pale yellow and transparent liquid

3. Confirmation of the Test Substance

The infrared (IR) spectrum of the test substance (NCAS retrieval No.: STD-1180-4) was measured (using KBr pellet) at the start of the experiment. This spectrum was compared with the IR spectrum obtained from the sponsor to confirm the identity.

4. Stability of the Test Substance

The IR spectrum of the test substance (NCAS retrieval No.: STD-1180-4) was measured at the end of the experiment. This spectrum was compared with the IR spectrum measured at the start of the experiment to confirm the stability.

5. Reagents and Apparatus

Potassium dihydrogenphosphate, disodium hydrogenphosphate dodecahydrate, ammonium chloride, magnesium sulfate heptahydrate, calcium chloride (anhydrous), iron (III) chloride hexahydrate and nitric acid: Guaranteed (Wako Pure Chemical Industries, Ltd.)  
Dipotassium hydrogenphosphate: Guaranteed (Junsei Chemical Co., Ltd.)  
Distilled water and acetonitrile: for HPLC (Wako Pure Chemical Industries, Ltd.)  
Soda lime: No.1 (Wako Pure Chemical Industries, Ltd.)  
Potassium bromide: KBr Powder (PIKE TECHNOLOGIES)  
Lithium standard solution: 1004 mg/L (Lot No. KWWJ3984, Expiry date: August 2011)  
Deionized water: Distilled water prepared with an automatic water distiller (WDA-15S, G-487, Isuzu Seisakusho Co., Ltd.) was purified with a pure water manufacturing device (LV-08, G-70, Toray Industries, Inc.).  
  
Balance: AG285 (G-549, Mettler-Toledo International Inc.)  
EB-330H (G-3, Shimadzu Corp.)  
Stirrer: IS-34N (G-440, IKEDA SCIENTIFIC Co., Ltd.)  
Stirrer/Hotplate (G-418, CORNING)  
Membrane filter: GL Chromatodisc (25A, 0.45 µm, KURABO INDUSTRIES LTD.)  
Micropipette: 500—5000 µL (MP-1-15, eppendorf Research)  
10—100 µL (MP-1-20, eppendorf Research)  
pH meter: 720A (G-677, Thermo Fisher Scientific K.K.)  
F-23 (G-254, HORIBA, Ltd.)  
Centrifuge: H-9R (G-674, KOKUSAN Co., Ltd.)  
Drier (for MLSS measurement): DO-300A (G-822, AS ONE Corp.)  
Standard thermometer: 0—50°C (G-588-77, ANDO KEIKI CO., LTD.)

Enclosed respirometer: Incubator OM-3100A (G-281, Ohkura Electric Co., Ltd.)  
 Recorder RM18G (G-281-1, Ohkura Electric Co., Ltd.)  
 Liquid chromatograph mass spectrometer (LC/MS/MS): Acquity/Q micro (G-722, Waters)  
 Atomic absorption spectrophotometer (AA): AA 240 (G-708, Varian, Inc.)  
 Infrared spectrophotometer: Spectrum One (G-641, PerkinElmer Inc.)

## 6. Experimental Methods

Biological oxygen demand (BOD) which occurred by the degradation of the test substance in the presence of the standard activated sludge was measured periodically (28 days) with the enclosed respirometer. After the last measurement of BOD, the concentration of the test substance was determined with LC/MS/MS and AA. And then the biodegradability of the test substance was evaluated from the results of the BOD and the concentration of the test substance.

### 6.1 Conditions of the Biodegradation Test

#### 6.1.1 Standard Activated Sludge

Supplier: Chemicals Evaluation and Research Institute, Japan  
 Received date: October 14, 2010  
 Lot No.: 201009R  
 Suspended solid concentration (MLSS): 3100 mg/L (October 18, 2010)

#### 6.1.2 Conditions

Temperature: 24.7 — 24.9°C (actual measurement)  
 Exposure period: 28 days (BOD measurement)  
 Volume: 300 mL  
 Concentrations: Test substance and aniline (standard substance) 100 mg/L  
 Standard activated sludge (MLSS) 30 mg/L

#### 6.1.3 Experimental Design and Addition of the Test Substance

Bottle No. 1: [Water + Test substance] system  
 Deionized water (300 mL) was transferred into a bottle and then the test substance (30 mg) was added to the water.

Bottle No. 2–No. 4: [Sludge + Test substance] system  
 Mineral medium\* (297.1 mL) was transferred into 3 bottles each and then 30 mg of the test substance was added into each bottle, followed by the addition of the standard activated sludge\*\* (2.9 mL) to each bottle.

Bottle No. 5: [Sludge + Aniline] system  
 Mineral medium\* (297.1 mL) was transferred into a bottle and then 29 µL (30 mg) of aniline was added into the bottle with a micropipette. After mixing it well, the standard activated sludge\*\* (2.9 mL) was added to the bottle.

Bottle No. 6: [Sludge blank] system  
 Mineral medium\* (297.1 mL) was transferred into a bottle and the standard activated sludge\*\* (2.9 mL) was added to the medium.

\*) A, B, C and D solutions (each 6 mL, defined in JIS K 0102-1998 21) were added to deionized water to make a 2-L solution. The addition volume of the mineral medium was calculated by the following equation.

$$V_m = 300 - V_s$$

where,  $V_m$  = Addition volume of the mineral medium (mL)

300 = Volume of the test solution (mL)

$V_s$  = Addition volume of the standard activated sludge (mL)

- \*\*) The addition volume of the standard activated sludge was calculated by the following equation based on the MLSS value.

$$V_s = C_s / \text{MLSS} \times V_o$$

where,  $V_s$  = Addition volume of the standard activated sludge (mL)

$C_s$  = Sludge concentration in the test solution (mg/L)

MLSS = Suspended solid concentration (mg/L, see section 0.1))

$V_o$  = Volume of the test solution (mL)

## 6.2 BOD Measurement and Observation of the Bottle Contents

BOD of each bottle was measured for 28 days with the enclosed respirometer. The growth of the activated sludge and color of the test systems were observed during the exposure period (after 7, 14, 21 and 28 days from the experimental start). The BOD degradability was calculated by the following equation (7, 14, 21 and 28 days).

$$\text{Degradability (\%)} = \{(\text{BOD} - B) / \text{TOD}\} \times 100$$

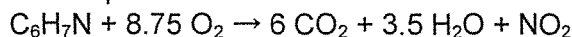
where, BOD: Oxygen demand in the [Sludge + Aniline] or [Sludge + Test substance] systems (mg)

B: Oxygen demand in the [Sludge blank] system (mg)

TOD: Theoretical oxygen demand of the test substance or aniline (mg)

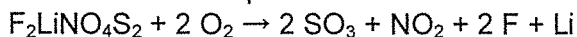
### <Calculation of the TOD>

Aniline: TOD was calculated by having assumed that 30 mg of aniline was decomposed as follows.



$$\text{TOD} = (30 / 93.13) \times 8.75 \times 32 = 90.2 \text{ mg}$$

Test substance: TOD was calculated by having assumed that 30 mg of the test substance was decomposed as follows.



$$\text{TOD} = (30 / 187.07) \times 2 \times 32 = 10.3 \text{ mg}$$

## 6.3 pH Measurement

The pH of the test solutions was measured at the start and the end of the exposure. For the [Water + Test substance] and the [Sludge + Test substance] systems, another solution for each system was prepared and the pH measurement of these solutions was conducted at the start of the exposure. At the end of the exposure, the pH was measured using the test solution which remained in the bottle after the treatment according to section 6.4.

## 6.4 Analytical Procedures after the End of the BOD Measurement

Figure 1 shows the outline of the analytical procedure after the end of the BOD measurement. An aliquot (100 mL) of each solution (bottle No. 1 to No. 4) was taken into a centrifugal tube and then it was centrifuged for 15 minutes at 5000 rpm (3944 g). The supernatant was filtered with a membrane filter and the filtrate was analyzed in accordance with the procedures described in sections 6.5 and 6.6.

For bottle No. 5, the above-mentioned operation was not carried out since only pH was measured.

## 6.5 LC/MS/MS Measurement

The concentration of the test substance in the test solution was determined in accordance with the procedure described in the next page.

### 6.5.1 LC/MS/MS Conditions

Column: Ascentis Express C18, 2.1 mm i.d. × 100 mm, 2.7 μm (SUPELCO)  
Column temperature: 40°C  
Mobile phase: Distilled water + acetonitrile = 1 + 1 (v/v, 0.1 v/v% formic acid was contained)  
Flow rate: 0.3 mL/min  
Injection volume: 5 μL  
Ionization mode: ESI, negative  
Measuring mode: MRM  
Monitored ion: 180.33>96.90 (for quantification), 180.33>77.84 (for confirmation)

### 6.5.2 Preparation of Calibration Curve

An aliquot (10.69 mg) of the test substance was dissolved in distilled water to make 100 mL solution. This solution was diluted with 50% acetonitrile in water to prepare 0.03, 1, 3 and 5 μg/mL calibration solutions. These calibration solutions (0.03 - 5 μg/mL) and 50% acetonitrile in water were injected into the LC/MS/MS described in section 6.5.1 in order to determine the peak areas of the test substance. The calibration curve was prepared by plotting the peak areas vs. the concentrations of the test substance to confirm the linearity. It was considered that the linearity of the calibration curve was good when the square of the correlation coefficient ( $R^2$ ) was more than 0.99.

### 6.5.3 Measurement of the Test Solutions

The test solutions (bottle Nos. 1, 2, 3 and 4) were treated in accordance with section 6.4 and 1.5 mL of each solution was diluted with 50% acetonitrile in water to make 50 mL solution. The diluted solutions were measured on the LC/MS/MS conditions described in section 6.5.1 ( $n=1$ ) and the peak areas of the test substance were determined. The concentrations of the test substance in the test solutions were calculated by using the calibration curve.

### 6.5.4 Recovery Test

The recovery of the analytical method was not carried out because no extraction procedure was included in the analytical procedure described in section 6.5.3.

### 6.5.5 Calculation of the Degradability

The amount of the residual test substance was calculated from the concentration of the test substance in the test solutions obtained from section 6.5.3. The degradability was calculated by the following equation.

$$\text{Degradability (\%)} = \{(C_B - C_A) / C_B\} \times 100$$

where,  $C_A$  = Amount of the residual test substance in the [Sludge + Test substance] system (mg)

$C_B$  = Amount of the residual test substance in the [Water + Test substance] system (mg)

### 6.5.6 Limit of Quantification

The lowest concentration (0.03 μg/mL) of the calibration solution prepared by section 6.5.2 was regarded as the limit of quantification. This concentration is equivalent to 1 μg/mL as the concentration of the test substance in the test solutions.

## 6.6 AA Measurement

The concentration of lithium in the test solutions was determined in accordance with the procedure described in the next page.

#### 6.6.1 AA Conditions

Hollow cathode lamp:	Lithium
Fuel / Support :	Air / acetylene
Electric current:	5.0 mA
Flow rate of air:	13.5 L/min
Flow rate of acetylene:	2.00 L/min
Wavelength:	670.8 nm
Slit width:	1.0 nm
Burner height:	0.0 mm
Number of replications:	5
Measuring time:	3 seconds

#### 6.6.2 Preparation of Calibration Curve

A lithium standard solution (1004 mg/L) was diluted with 0.2 mol/L nitric acid to prepare 0.08, 0.2, 0.4, 0.8, 1.2 and 2 mg/L calibration solutions. These calibration solutions (0.08 - 2 mg/L) were injected into the AA conditions described in section 6.6.1 and the mean absorbance of five repetitive measurements was calculated. The calibration curve was prepared by plotting the mean absorbance vs. the concentrations of the test substance to confirm the linearity. It was considered that the linearity of the calibration curve was good when the square of the correlation coefficient ( $R^2$ ) was more than 0.99.

#### 6.6.3 Measurement of the Test Solutions

The test solutions (bottle Nos. 1, 2, 3 and 4) were treated in accordance with section 6.4. Aliquots of the each solution (4 mL) and 0.4 mol/L nitric acid (4 mL) were taken into a 20-mL volumetric flask. In addition, they were diluted with 0.2 mol/L nitric acid. The diluted solutions were measured on the AA conditions described in section 6.6.1 ( $n=1$ ) and the mean absorbance of five repetitive measurements was calculated. The concentrations of lithium in the test solutions were calculated by using the calibration curve.

#### 6.6.4 Calculation of the Degradability

The amount of the residual lithium was calculated from the concentration of lithium in the test solutions obtained from section 6.6.3. The degradability was calculated by a following equation.

$$\text{Degradability (\%)} = \{(C_B - C_A) / C_B\} \times 100$$

where,  $C_A$  = Amount of the residual lithium in the [Sludge + Test substance] system (mg)

$C_B$  = Amount of the residual lithium in the [Water + Test substance] system (mg)

#### 6.7 Measurement of the Dissolved Organic Carbon

The measurement of the amount of the dissolved organic carbon was not carried out since no carbon was contained in the test substance.

## Results and Discussion

### 1. Confirmation of the Test Substance

Figure 2 shows the IR spectrum of \_\_\_\_\_ supplied from the sponsor and Figure 3 shows the IR spectrum of the test substance (NCAS retrieval No. STD-1180-4) measured before the experiment.

Since both IR spectra coincided with each other, it was considered that the test substance was \_\_\_\_\_. However, the peak width became large because the test substance had deliquescence. Furthermore, IR spectrum of another test substance (NCAS retrieval No. STD-1180-3, the same lot) was measured and the same spectrum as Figure 3 was obtained. Therefore, no difference was recognized on the property of the test substance among the NCAS retrieval Nos. Thus, the calibration solutions for LC/MS/MS were prepared by using NCAS retrieval No. STD-1180-6 which had the same lot number the others.

### 2. Confirmation of the Stability of the Test Substance

Figure 4 shows the IR spectrum of the test substance measured after the experiment.

The IR spectra obtained before and after the experiment coincided with each other, so that it was considered that the test substance was stable during the experimental period under room temperature (dark place).

### 3. Confirmation of the Degradation Activity of the Activated Sludge

Table 1 ([Sludge + Aniline] system) shows the results of the BOD degradability of aniline to confirm the degradation activity of the activated sludge. The BOD degradabilities of aniline were 51% (7 days after the exposure) and 65% (14 days after the exposure). Therefore, it was considered that the activated sludge had good activity. Thus, the validity of this study was confirmed. By the way, the activated sludge of another lot (lot No.: 201006R, received date: July 15, 2010) was tested before using the activated sludge of this lot. However, BOD degradability was less than 40% after 7 days. Thus, it was considered that this test was invalid.

### 4. Observation Results of the Contents

Table 2 shows the observation results of the contents. The test solutions of the [Sludge + Test substance] system (Bottle Nos. 2 - 4) and the [Sludge blank] system (Bottle No. 6) were slightly muddy white. The test solution of the [Sludge + Aniline] system (Bottle No. 5) was muddy white and that of the [Water + Test substance] system (Bottle No. 1) was colorless and transparent. The multiplication of activated sludge was observed in the [sludge + aniline] system only.

### 5. Results of the pH Measurement

Table 3 shows the pH values measured at the start and end of the cultivation.

At the start of the cultivation, the pH values of the [Water + Test substance] system, the [Sludge + Test substance] system, the [Sludge + Aniline] system and the [Sludge blank] system solutions were 5.3, 7.3, 7.3 and 7.3, respectively. At the end of the cultivation, the pH values of each system solution were 6.4, 7.3 (mean value), 8.4 and 7.7, respectively.

#### 6. Degradability Based on the BOD Measurement

Table 1 shows the degradability based on the BOD measurement and Figures 5 to 8 show the charts of the BOD measurement.

The BOD values after 28 days for the [Sludge + Test substance] system were 1.9380 mg for bottle No. 2, 2.0440 mg for bottle No. 3 and 1.9960 mg for bottle No. 4. The BOD values for the [Sludge blank] and [Water + Test substance] systems were 1.7450 mg and 0.0000 mg, respectively. Since the TOD of the test substance was 10.3 mg, the BOD degradabilities of the [Sludge + Test substance] system were 2% for bottle No. 2, 3% for bottle No. 3 and 2% for bottle No. 4. Therefore, the mean degradability was calculated to be 2%. The difference of the degradation rates between maximum and minimum was less than 20% so the validity criterion of the biodegradation test was fulfilled.

#### 7. Degradability Based on the LC/MS/MS Measurement

Table 4 shows the degradability based on the LC/MS/MS measurement. Figure 9 shows the calibration curve and Figures 10 to 17 show the LC/MS/MS chromatograms of the standard solutions and the test samples.

The amounts of the residual test substance in the [Sludge + Test substance] system were 31.1 mg for bottle No. 2, 29.5 mg for bottle No. 3 and 30.9 mg for bottle No. 4. The amount of the residual test substance in the [Water + Test substance] system was 30.4 mg. The degradation rates of the [Sludge + Test substance] system were -2% for bottle No. 2, 3% for bottle No. 3 and -2% for bottle No. 4. Therefore, the mean degradability was calculated as 0%. The difference of the degradation rates between maximum and minimum was less than 20% so the validity criterion of the biodegradation test was fulfilled.

No unknown peak which had larger area than the LOQ was observed on the LC/MS/MS chromatogram of the [Sludge + Test substance] system.

#### 8. Degradability Based on the AA Measurement

Table 5 shows the degradability based on the AA measurement, and Figure 18 shows the calibration curve.

The amounts of the residual lithium in the [Sludge + Test substance] system were 1.13 mg for bottle No. 2, 1.09 mg for bottle No. 3 and 1.12 mg for bottle No. 4. The amount of the residual lithium in the [Water + Test substance] system was 1.07 mg. The degradation rates of the [Sludge + Test substance] system were -6% for bottle No. 2, -2% for bottle No. 3 and -5% for bottle No. 4. Therefore, the mean degradability was calculated as -4%. The difference of the degradation rates between maximum and minimum was less than 20% so the validity criterion of the biodegradation test was fulfilled.

#### 9. Statistical Work

For the LC/MS/MS measurement, the concentration of the test substance in the test solutions were calculated by using the LC/MS/MS software and the degradabilities were calculated by using the Microsoft Excel 2003 (SP3). The degradability was rounded off by the first decimal place.

## Conclusions

The biodegradation test of (28 days) was conducted. The mean BOD degradability was 2% (n=3). The mean degradabilities calculated from the results of the LC/MS/MS and AA measurements were 0% (n=3) and -4% (n=3), respectively. Therefore, it was considered that is not ready biodegradable because the degradabilities for all vessels were less than 60% (Test Methods and Judgment Criteria for the Designation of Monitoring Chemical Substances, April 12, 2010).



Table 1 Degradability of the test substance based on the BOD measurement

Bottle No.	Sample	7 days		14 days		21 days		28 days		
		BOD (mg)	DG* (%)	BOD (mg)	DG* (%)	BOD (mg)	DG* (%)	BOD (mg)	DG* (%)	Mean (%)
1	Water + Test substance	0.000	—	0.000	—	0.000	—	0.0000	—	—
2	Sludge + Test substance	0.594	2	1.653	3	1.938	5	1.9380	2	2
3	Sludge + Test substance	0.256	-1	1.391	0	1.822	3	2.0440	3	
4	Sludge + Test substance	0.571	2	1.590	2	1.829	3	1.9960	2	
5	Sludge + Aniline	46.09	51	59.94	65	62.17	67	64.193	69	—
6	Sludge blank	0.353	—	1.389	—	1.473	—	1.7450	—	—

\*\* : DG (Degradability, %) =  $\{(BOD - B) / TOD\} \times 100$

BOD: Oxygen demand in the [Sludge + Aniline] or [Sludge + Test substance] systems (mg)

B: Oxygen demand in the [Sludge blank] system (mg)

TOD: Theoretical oxygen demand (aniline = 90.2 mg, test substance = 10.3 mg)

Table 2 Observation results of the contents

Bottle No.	Sample	7days		14 days		21 days		28 days	
		Proliferation	Appearance	Proliferation	Appearance	Proliferation	Appearance	Proliferation	Appearance
1	Water + Test substance	—	colorless transparency	—	colorless transparency	—	colorless transparency	—	colorless transparency
2	Sludge + Test substance	None	slightly muddy white	None	slightly muddy white	None	slightly muddy white	None	slightly muddy white
3	Sludge + Test substance	None	slightly muddy white	None	slightly muddy white	None	slightly muddy white	None	slightly muddy white
4	Sludge + Test substance	None	slightly muddy white	None	slightly muddy white	None	slightly muddy white	None	slightly muddy white
5	Sludge + Aniline	observed	muddy white	observed	muddy white	observed	muddy white	observed	muddy white
6	Sludge blank	None	slightly muddy white	None	slightly muddy white	None	slightly muddy white	None	slightly muddy white

Table 3 pH values of the test solutions

Bottle No.	Sample	pH		
		at the start	at the end	
			Individual	Mean
1	Water + Test substance	5.3	6.4	—
2	Sludge + Test substance	7.3	7.2	7.3
3	Sludge + Test substance		7.4	
4	Sludge + Test substance		7.4	
5	Sludge + Aniline	7.3	8.4	—
6	Sludge blank	7.3	7.7	—

Table 4 Degradability of the test substance based on the LC/MS/MS measurement

Bottle No.	Sample	Peak Area	Concentration (mg/L)		Residual amount (mg)	Degradability (%)	
			Measured solution	Test solution		Individual	Mean
1	Water + Test substance	1671.6	3.0357	101.2	30.4	—	—
2	Sludge + Test substance	1713.5	3.1134	103.8	31.1	-2	0
3	Sludge + Test substance	1626.0	2.9508	98.36	29.5	3	
4	Sludge + Test substance	1703.3	3.0944	103.1	30.9	-2	

Calibration equation:  $y = 538.749x + 36.1851$ ,  $R^2 = 0.999247$

$$\text{Degradability (\%)} = \{(C_B - C_A) / C_B\} \times 100$$

where,  $C_A$ : Amount of the residual test substance in the [Sludge + Test substance] system (mg)

$C_B$ : Amount of the residual test substance in the [Water + Test substance] system (mg)

Table 5 Degradability of the test substance based on the AA measurement

Bottle No.	Sample	Absorbance (Abs)	Concentration of Li (mg/L)		Residential amount of Li (mg)	Degradability (%)	
			Measured solution	Test solution		Individual	Mean
1	Water + Test substance	0.2548	0.7158	3.5791	1.07	—	—
2	Sludge + Test substance	0.2673	0.7529	3.7644	1.13	-6	-4
3	Sludge + Test substance	0.2588	0.7277	3.6384	1.09	-2	
4	Sludge + Test substance	0.2644	0.7443	3.7214	1.12	-5	

Calibration equation:  $y = 0.3373x + 0.0133$ ,  $R^2 = 0.9985$

$$\text{Degradability (\%)} = \{(C_B - C_A) / C_B\} \times 100$$

where,  $C_A$ : Amount of the residual lithium in the [Sludge + Test substance] system (mg)

$C_B$ : Amount of the residual lithium in the [Water + Test substance] system (mg)

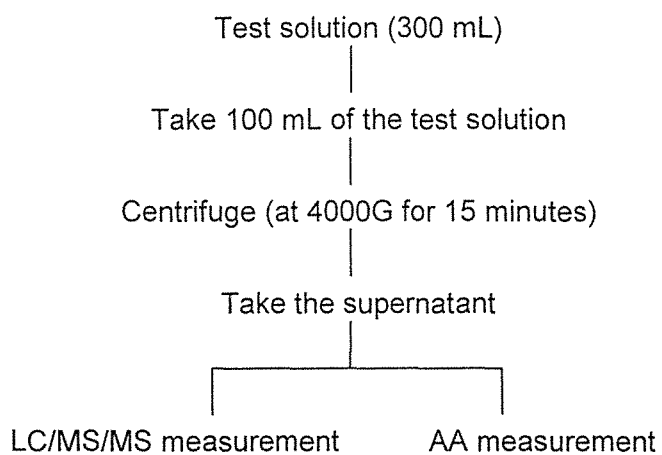


Figure 1 Outline of the analytical procedure after the BOD measurement

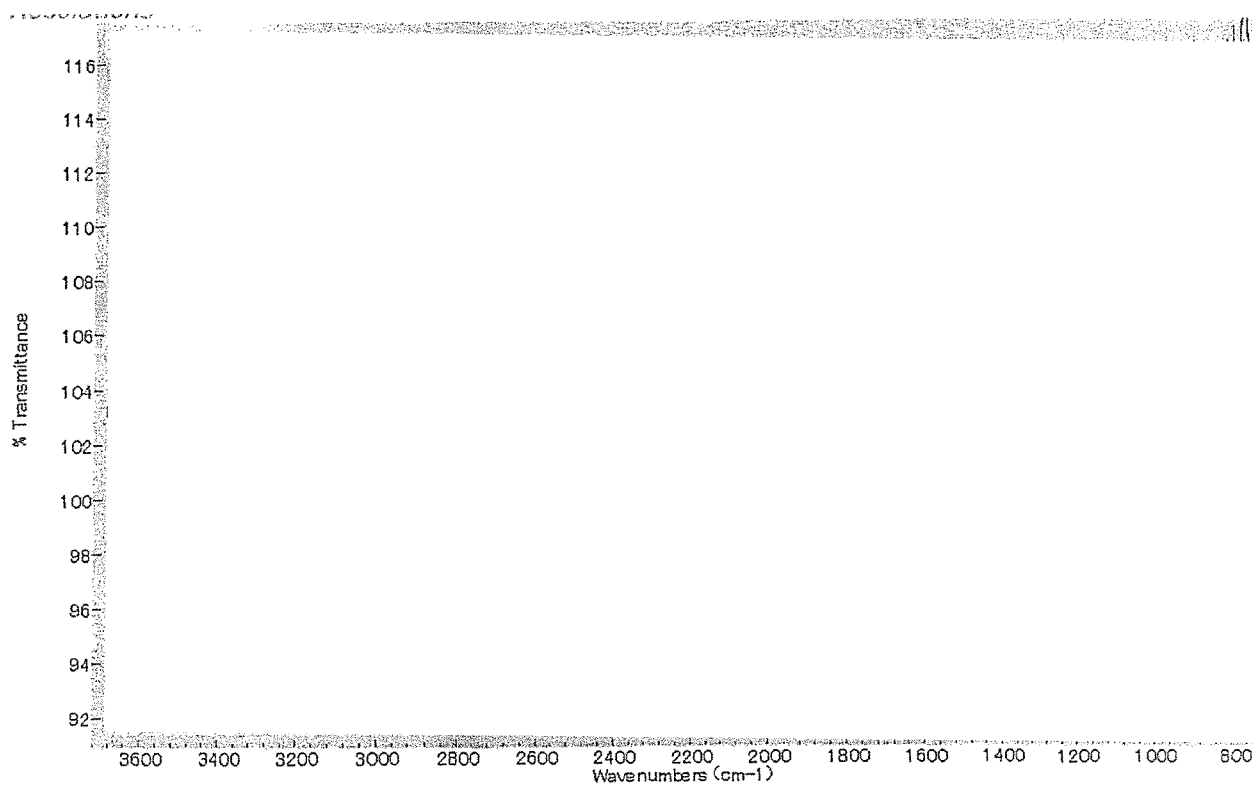


Figure 2 IR spectrum of the test substance (supplied from the sponsor)

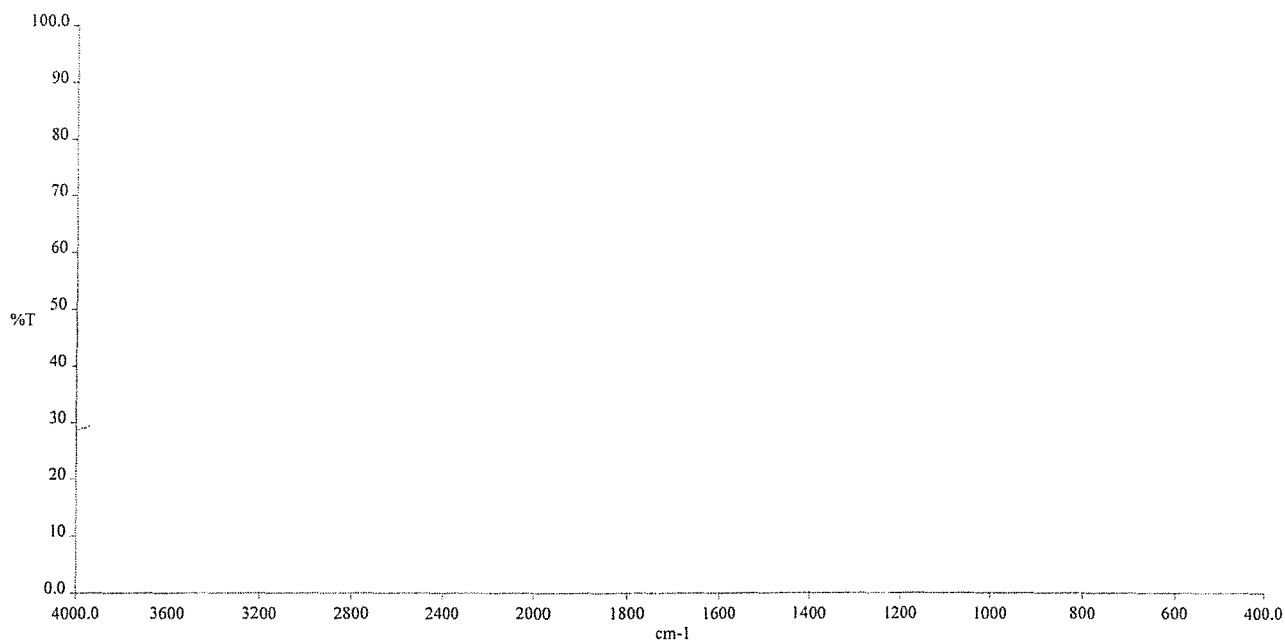


Figure 3 IR spectrum of the test substance measured before the experiment

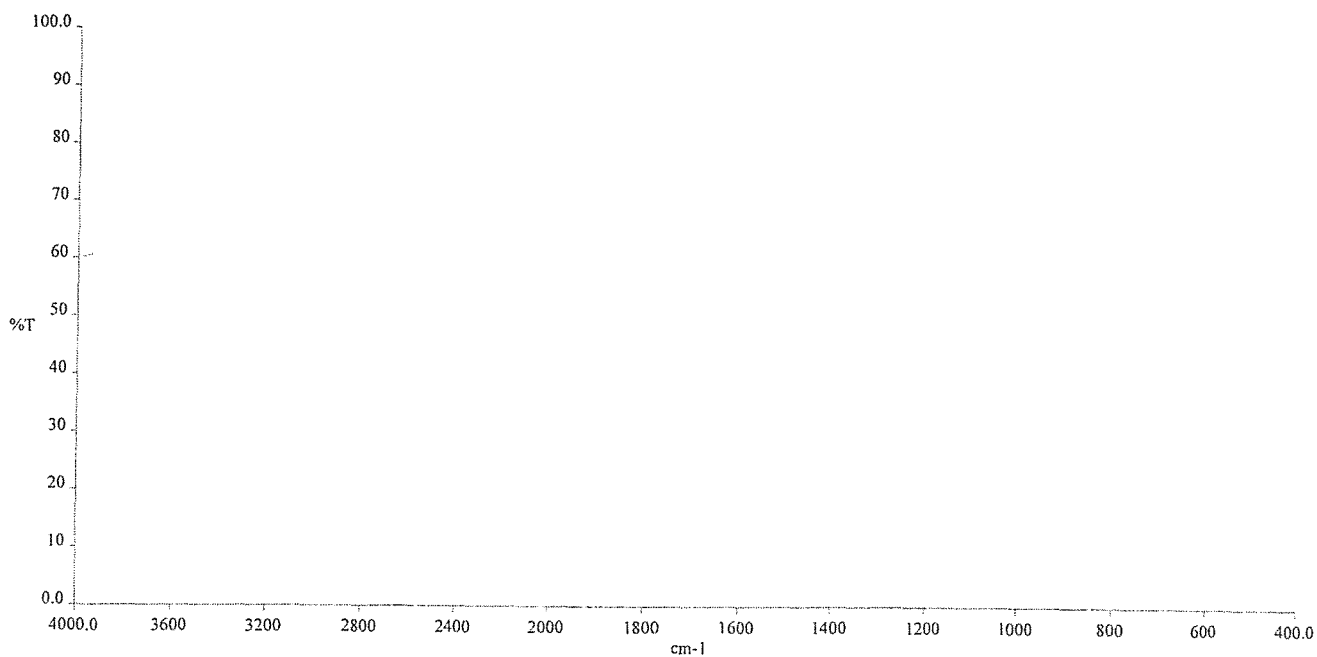


Figure 4 IR spectrum of the test substance measured after the experiment

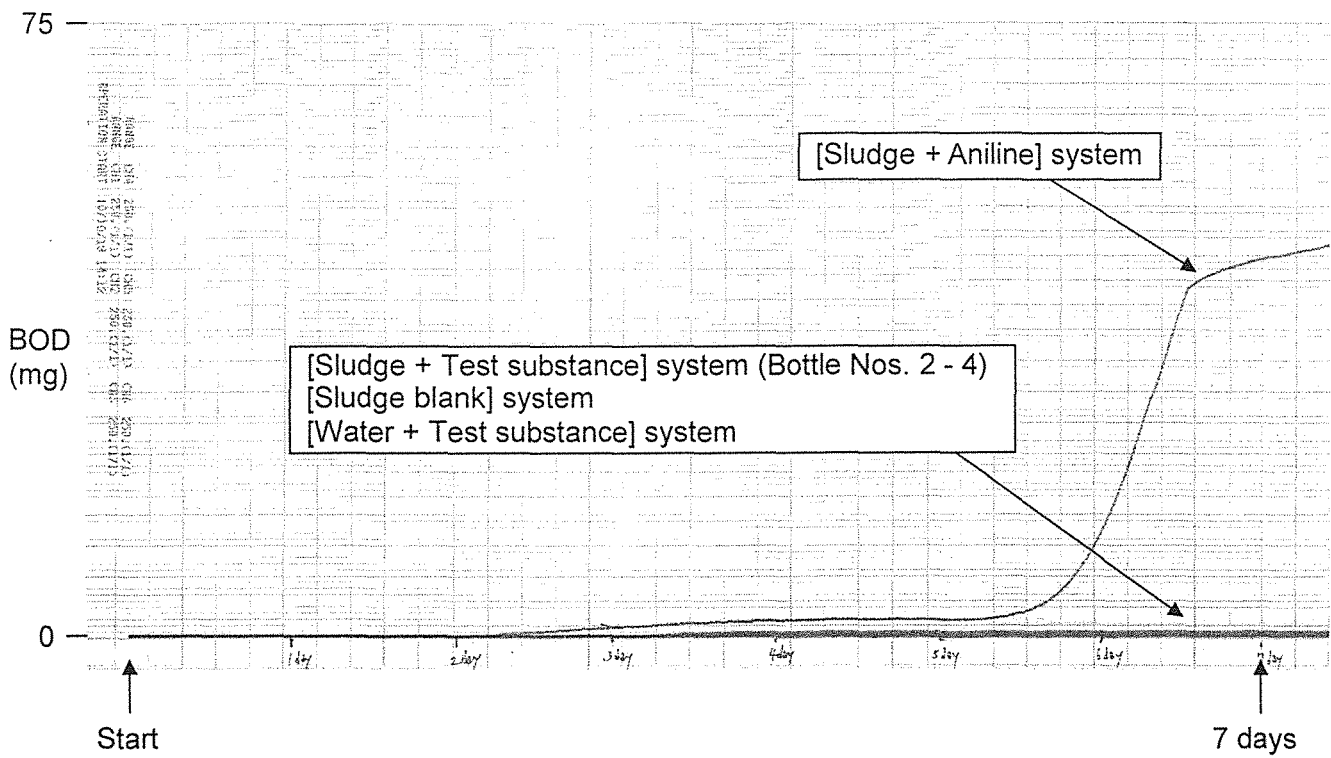


Figure 5 BOD measuring chart (start - 7 days)

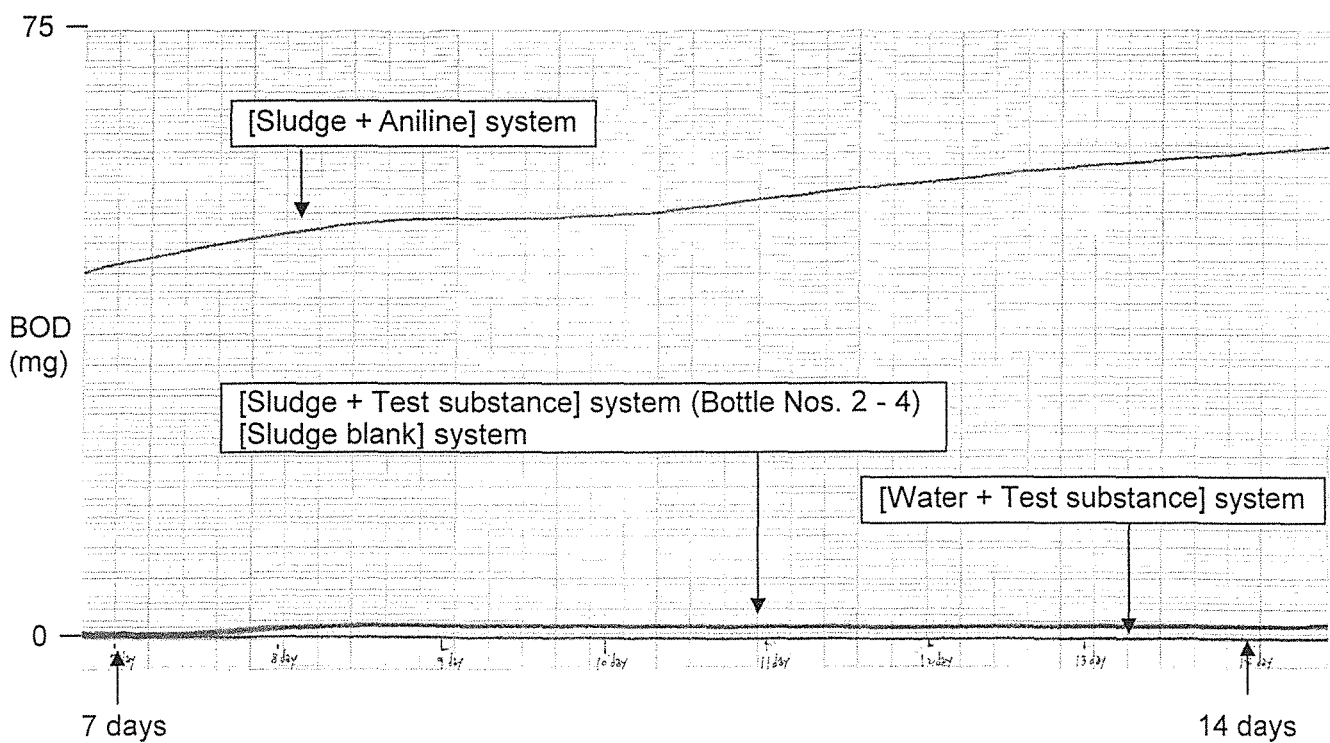


Figure 6 BOD measuring chart (7 days - 14 days)

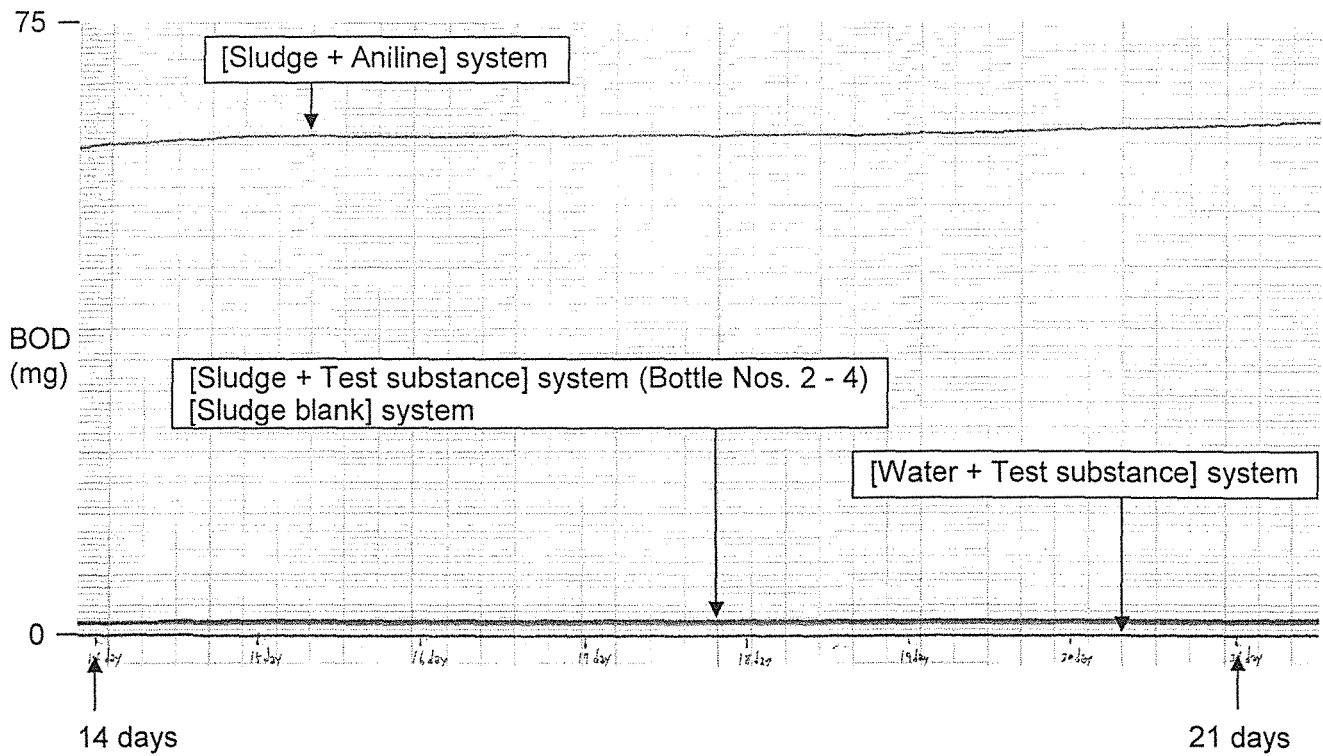


Figure 7 BOD measuring chart (14 days - 21 days)

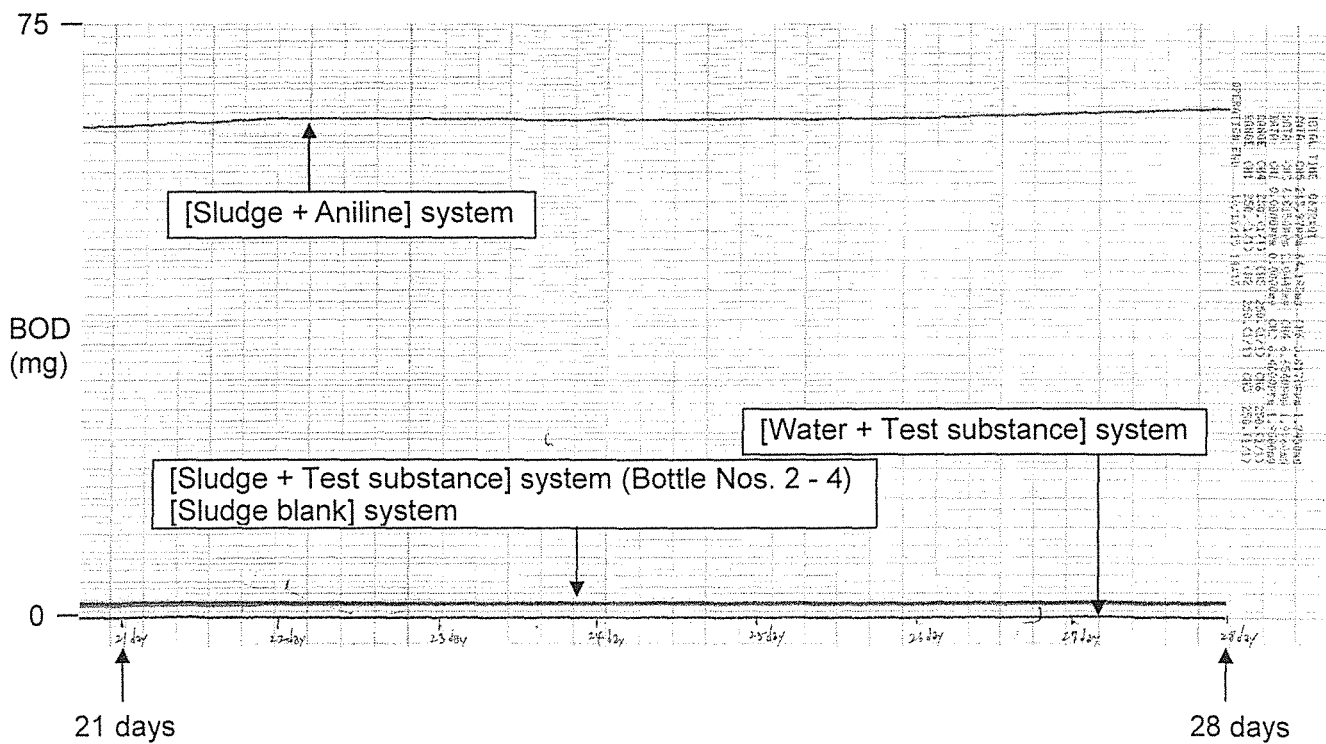


Figure 8 BOD measuring chart (21 days - 28 days)

Compound name:

Correlation coefficient:  $r = 0.999624$ ,  $r^2 = 0.999247$

Calibration curve:  $538.749 * x + 36.1851$

Response type: External Std, Area

Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None

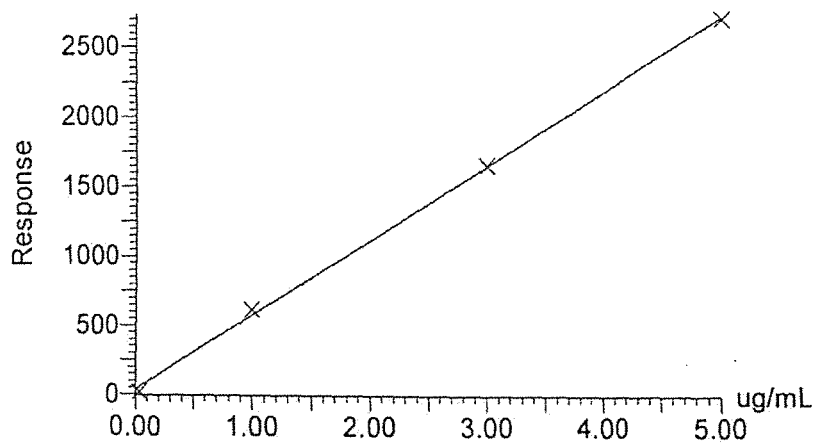
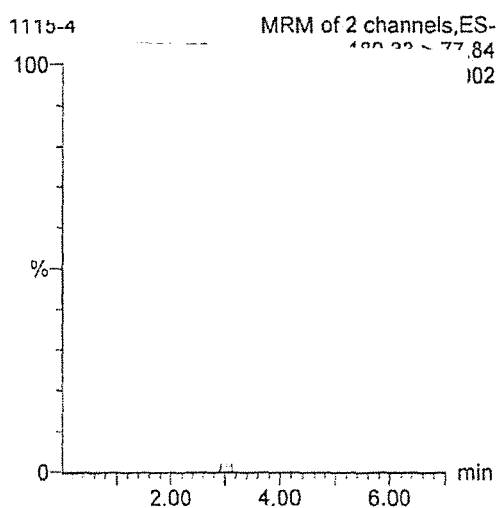
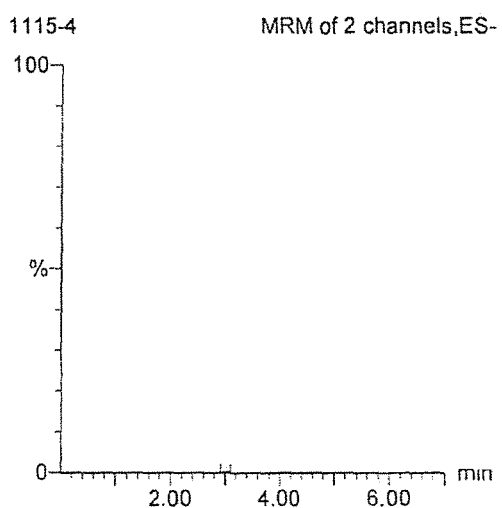


Figure 9 Calibration curve of the LC/MS/MS measurement

Name: 1115-4, Date: 15-Nov-2010, Time: 16:29:50, Description: STD 0.03 ug/mL



#	Name	Trace	RT	Area	ug/mL	%Rec

for quantification                      for qualification

Figure 10 LC/MS/MS chromatogram of the calibration solution at 0.03  $\mu$ g/mL

Name: 1115-5, Date: 15-Nov-2010, Time: 16:37:54, Description: STD 1.0 ug/mL

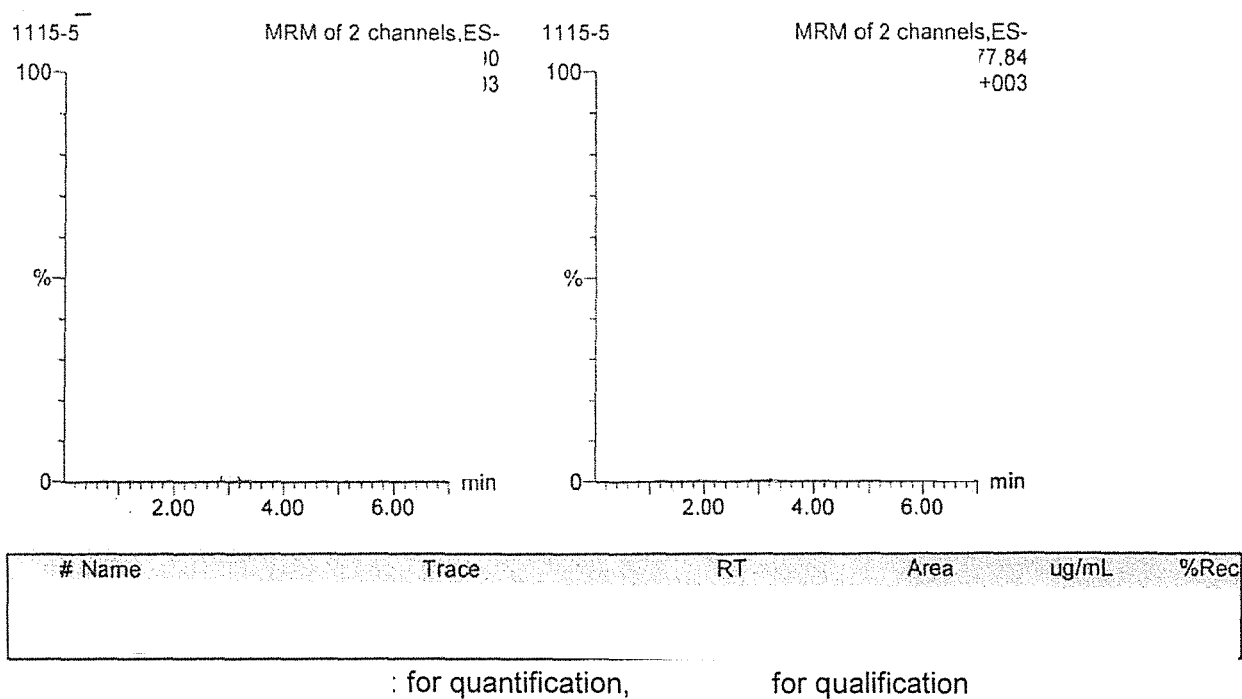


Figure 11 LC/MS/MS chromatogram of the calibration solution at 1 µg/mL

Name: 1115-6, Date: 15-Nov-2010, Time: 16:45:59, Description: STD 3.0 ug/mL

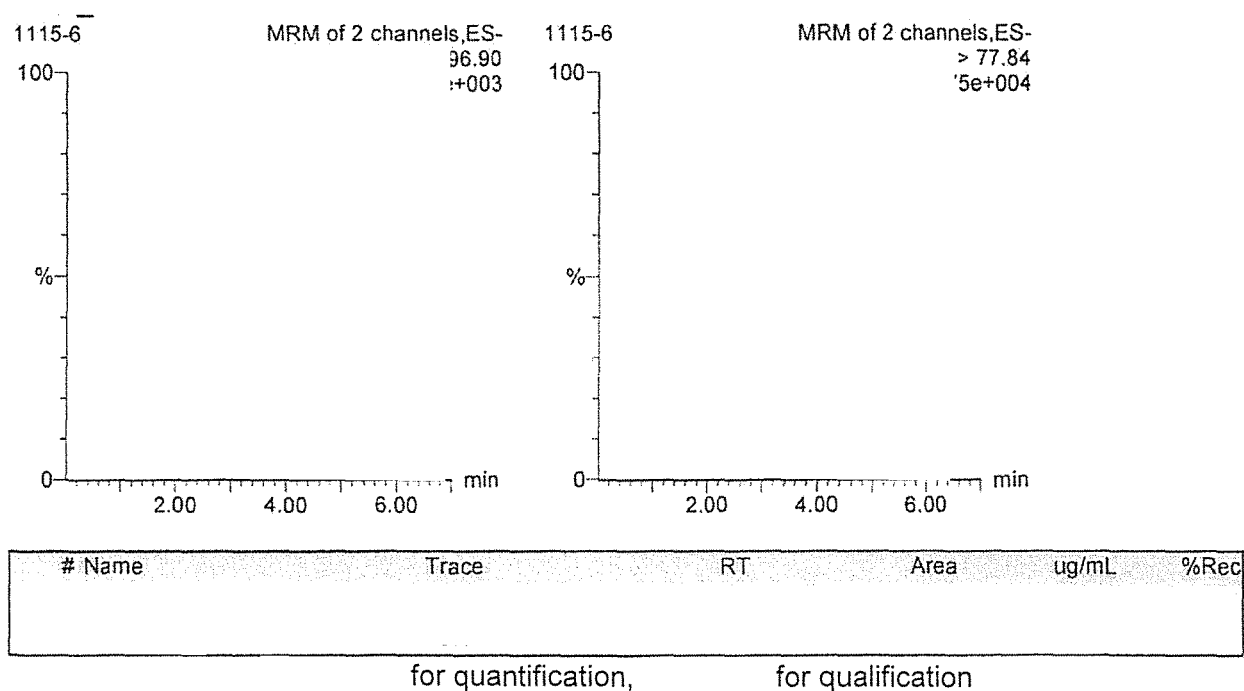


Figure 12 LC/MS/MS chromatogram of the calibration solution at 3 µg/mL



Name: 1115-7, Date: 15-Nov-2010, Time: 16:54:05, Description: STD 5.0 ug/mL

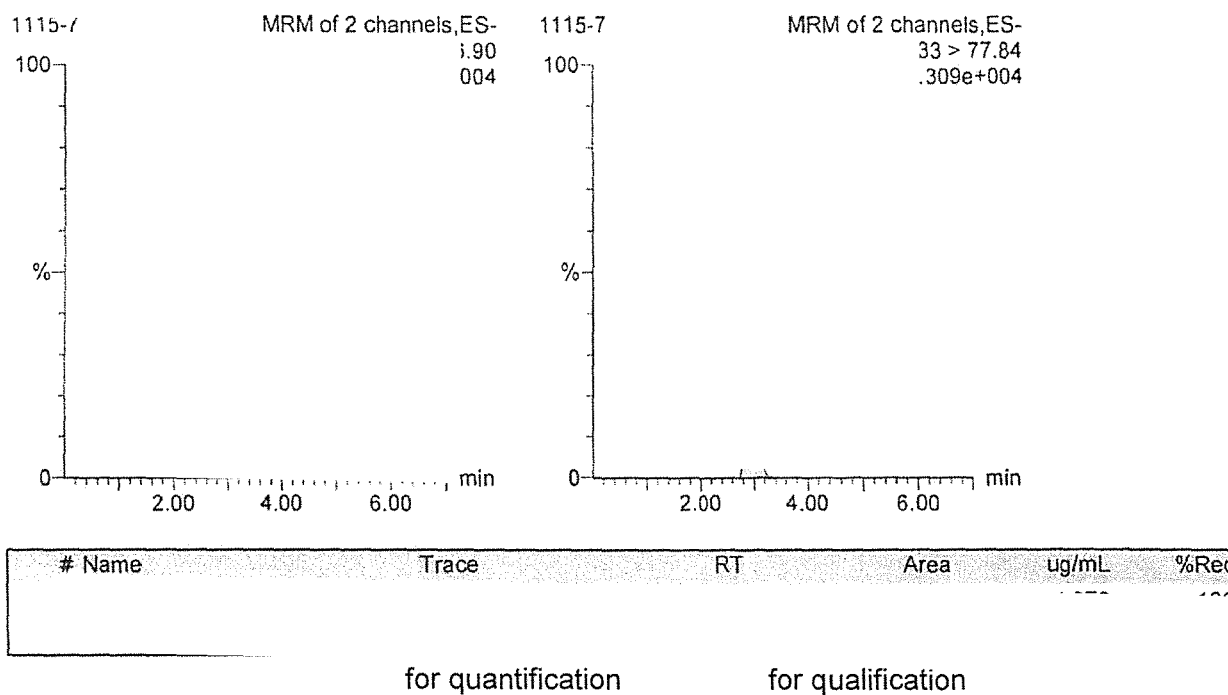
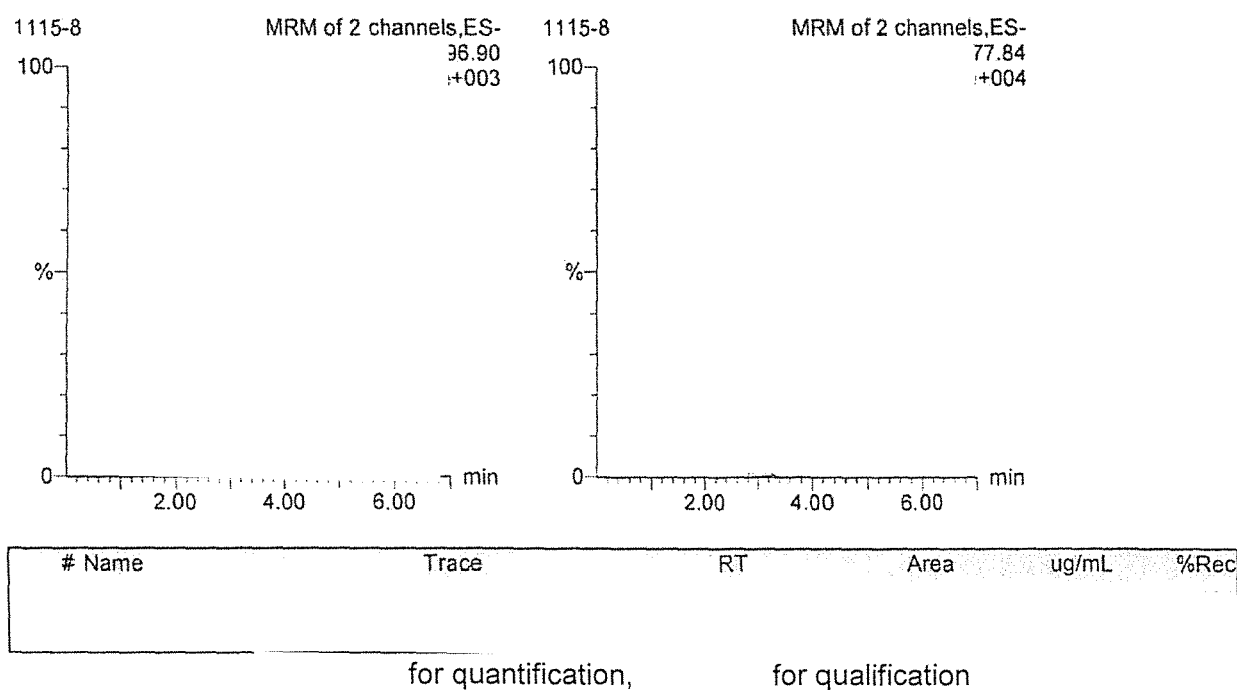


Figure 13 LC/MS/MS chromatogram of the calibration solution at 5 µg/mL

Name: 1115-8, Date: 15-Nov-2010, Time: 17:02:12, Description: No.1\_221Water T



Name: 1115-9, Date: 15-Nov-2010, Time: 17:10:16, Description: No.2\_221-1T

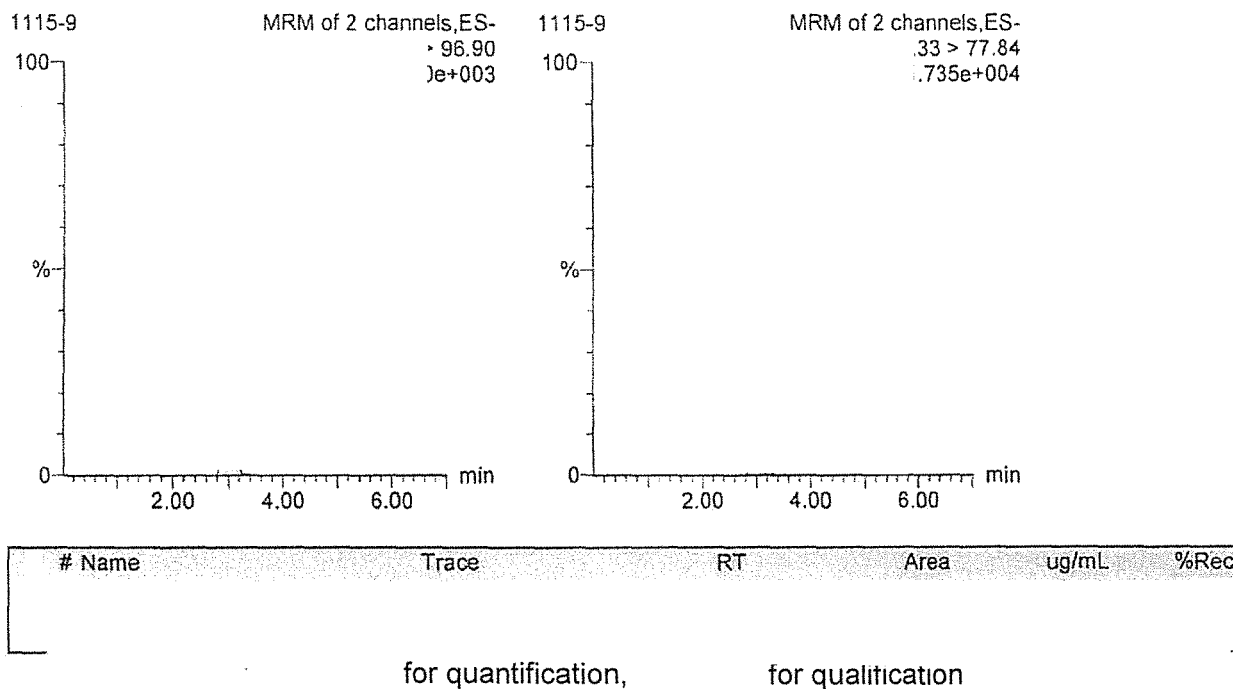


Figure 15 LC/MS/MS chromatogram of the test solution for the [Sludge + Test Substance] System (Bottle No. 2)

Name: 1115-10, Date: 15-Nov-2010, Time: 17:18:23, Description: No.3\_221-2T

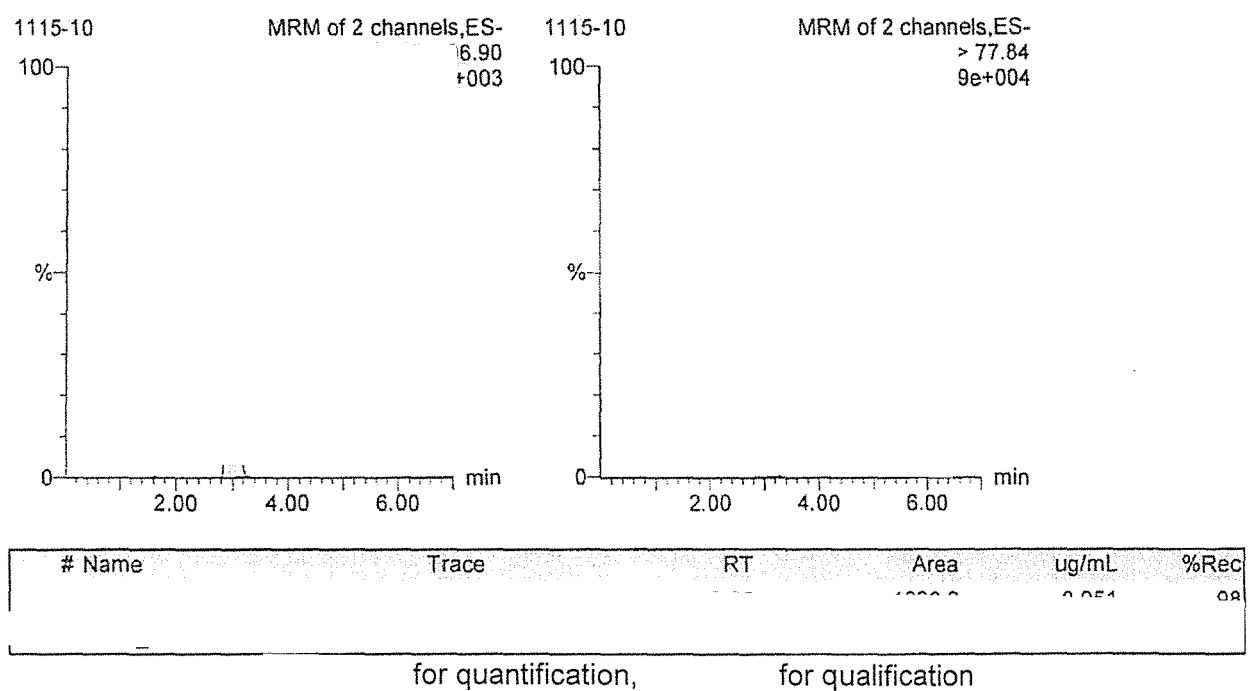
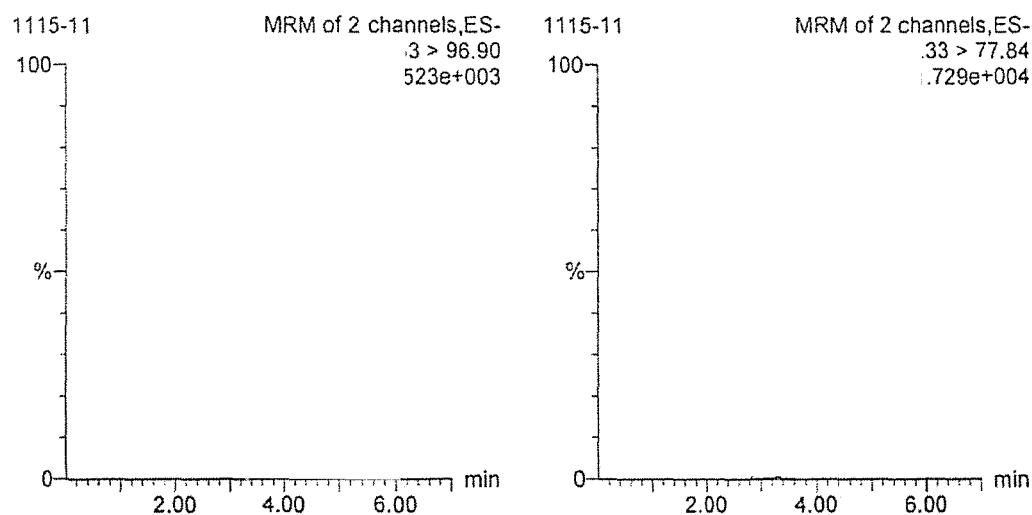


Figure 16 LC/MS/MS chromatogram of the test solution for the [Sludge + Test Substance] System (Bottle No. 3)

Name: 1115-11, Date: 15-Nov-2010, Time: 17:26:27, Description: No.4\_221-3T



#	Name	Trace	RT	Area	ug/mL	%Rec
1	1115-11		2.00			

for quantification, for qualification

Figure 17 LC/MS/MS chromatogram of the test solution for the [Sludge + Test Substance] System (Bottle No. 4)

濃度 (mg/L)	吸光度 (Abs)
0.08032	0.0308
0.2008	0.0766
0.4016	0.1522
0.8032	0.2944
1.205	0.4309
2.008	0.6801

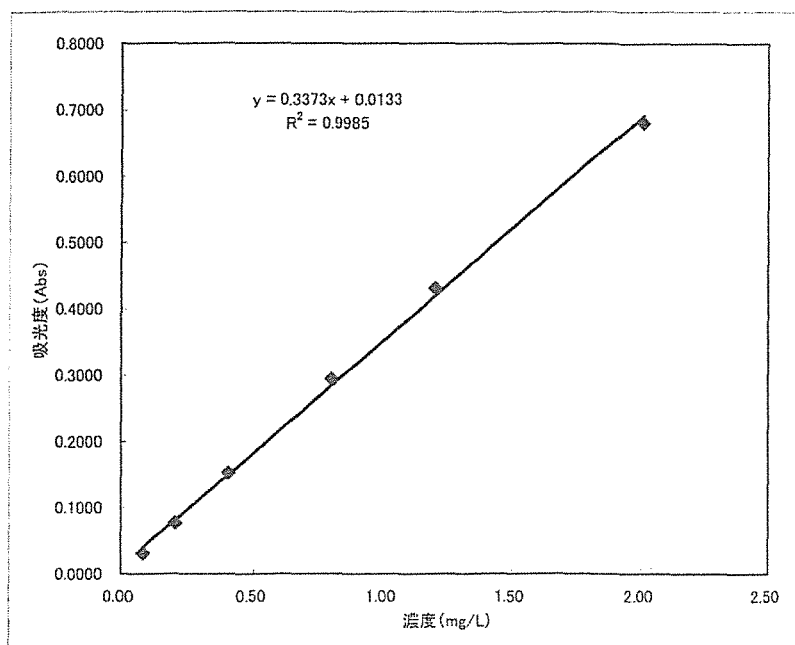


Figure 18 Calibration curve of the AA measurement

## Authenticity of Translation

I declare that the original Japanese final report (Report No. NCAS 10-221) is translated into English consistently.

Translated by :

Kenji Miya

Kenji Miya

Nisso Chemical Analysis Service Co., Ltd.

Odawara Laboratory

June 14, 2012

Date